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Early life stress elicits visceral hyperalgesia and functional reorganization of pain circuits in adult rats

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ABSTRACT

Early life stress (ELS) is a risk factor for developing functional gastrointestinal disorders, and has been proposed to be related to a central amplification of sensory input and resultant visceral hyperalgesia. We sought to characterize ELS-related changes in functional brain responses during acute noxious visceral stimulation. Neonatal rats (males/females) were exposed to limited bedding (ELS) or standard bedding (controls) on postnatal days 2–9. Age 10–11 weeks, animals were implanted with venous cannulas and transmitters for abdominal electromyography (EMG). Cerebral blood flow (rCBF) was mapped during colorectal distension (CRD) using [^{14}C]-iodoantipyrine autoradiography, and analyzed in three-dimensionally reconstructed brains by statistical parametric mapping and functional connectivity. EMG responses to CRD were increased after ELS, with no evidence of a sex difference. ELS rats compared to controls showed a greater significant positive correlation of EMG with amygdalar rCBF. Factorial analysis revealed a significant main effect of 'ELS' on functional activation of nodes within the pain pathway (somatosensory, insular, cingulate and prefrontal cortices, locus coeruleus/lateral parabrachial n. [LC/LPB], periaqueductal gray, sensory thalamus), as well as in the amygdala, hippocampus and hypothalamus. In addition, ELS resulted in an increase in the number of significant functional connections (i.e. degree centrality) between regions within the pain circuit, including the amygdala, LC/LPB, insula, anterior ventral cingulate, posterior cingulate (retrosplenium), and stria terminalis, with decreases noted in the sensory thalamus and the hippocampus. Sex differences in rCBF were less broadly expressed, with significant differences noted at the level of the cortex, amygdala, dorsal hippocampus, raphe, sensory thalamus, and caudate-putamen. ELS showed a sexually dimorphic effect ('Sex x ELS' interaction) at the LC/LPB complex, globus pallidus, hypothalamus, raphe, septum, caudate-putamen and cerebellum. Our results suggest that ELS alters functional

Abbreviations: Acb, Accumbens n; aIns, anterior insula; ANOVA, analysis of variance; cc, corpus callosum; La, lateral amygdala; CA1, CA1 field of hippocampus; Ce, amygdala, central n; Cg1, Cg2, cingulate cortex area 1 dorsal, area 2 ventral; CM, central medial thalamic n; CPu, dorsal caudate-putamen; CON, controls; CRD, colorectal distension; DG, dentate gyrus; dHPC, dorsal hippocampus; Ect/TeA, ectorhinal/temporal association cortex; ELS, early life stress; EMG, electromyography; FC, functional connectivity; GPe, globus pallidus, external; Hb, habenula; HPC, hippocampus; IBS, irritable bowel syndrome; IC, inferior colliculus; La, amygdala, lateral n; LC/LPB, locus coeruleus/lateral parabrachial n; ll, lateral lemniscus; LD, lateral dorsal thalamic n; LO/VO, lateral/ventral orbital cortex; LSI, lateral septum intermediate; M1/M2, primary/secondary motor cortex; MD, mediodorsal thalamic nucleus; Me, amygdala, medial n; MG, medial geniculate; MnR, median raphe n; MPA, hypothalamic medial preoptic area; PAG, periaqueductal gray; PBP, parabrachial pigmented n. of the ventral tegmental area (VTA); pIns, posterior insula; pIns, posterior insula; pIns/Ect, posterior insula/ectorhinal cortex transition area; Po, posterior thalamic n; PrL, prelimbic cortex; PtA, parietal association cortex; PVP, posterior paraventricular thalamic n; rCBF, regional cerebral blood flow; ROI, region of interest; RS, retrosplenial cortex, 'posterior cingulate'; S1BF / S1FL / S1HL / S1J / S1ULp, primary somatosensory cortex, barrel field / forelimb / hindlimb / jaw / upper lip area; S2, secondary somatosensory cortex; SC, superior colliculus; Sim, simple cerebellar lobule; SPM, statistical parametric mapping; STM, bed n. of the stria terminalis; V1/V2, primary/secondary visual cortex; VMH, ventromedial hypothalamus; VMR, visceromotor response; VPL/VPM, ventral-posterolateral/ventral-postereromedial thalamic n.

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activation of the thalamo-cortico-amygdala pathway, as well as the emotional-arousal network (amygdala, locus coeruleus), with evidence that ELS may additionally show sexually dimorphic effects on brain function.

1. Introduction

Increasing evidence suggests that patients with irritable bowel syndrome (IBS) are more likely than healthy subjects to have a history of physical or sexual abuse (Ross, 2005; Walker et al., 1993), trauma (Irwin et al., 1996), and stress (Levy et al., 1997; Son et al., 2008), and in particular early life stress (Bradford et al., 2012; Grad et al., 2014; Halland et al., 2014). In addition, IBS patients compared to control subjects show visceral perceptual alterations during acute (Dickhaus et al., 2003; Elsenbruch et al., 2010; Posserud et al., 2004; Walter et al., 2006) and chronic (Eriksson et al., 2008; Patacchioli et al., 2001) emotional stress. Such findings have prompted hypotheses that life stressors, including those experienced in the early period of life (Chitkara et al., 2008), may trigger long-term changes in brain structure, brain function and visceral sensitivity to noxious stimuli (Chitkara et al., 2008; Murray et al., 2004; Posserud et al., 2004). Based on this, it is believed that part of the symptomatology of patients with functional bowel disorders may be related to a central amplification of sensory input and resultant visceral hyperalgesia, a core symptom of patients with IBS.

Emotions are well known to impact visceral function, and emotional and visceral neuronal circuits are substantially coextensive (Berntson et al., 2003; Bienkowski and Rinaman, 2008; Cameron, 2001; Craig, 2002; King et al., 1999; Price, 1999; Saper, 2002; Thayer and Lane, 2000). A cause and effect relationship between early life stress and visceral hyperalgesia has not yet been established in human subjects. However, prospective studies in preclinical models suggest that raising animals in environments characterized by aberrant maternal care (e.g. neonatal maternal separation (Bian et al., 2010; Coutinho et al., 2002; Welting et al., 2005; Wouters et al., 2012), limited bedding (Guo et al., 2015; Prusator and Greenwood-Van Meerveld, 2015)), results in visceral hyperalgesia in the adult offspring. In rats, central pre-autonomic circuits undergo significant synaptic assembly during the first two weeks of postnatal life (Rinaman et al., 2011). Early life stress (ELS) appears to disrupt maturation of the assembly of limbic, hypothalamic and cortical inputs, to autonomic neurons, and it is hypothesized that this may result in aberrant sensory processing in adult life (Card et al., 2005).

In human subjects, ELS (also referred to as early life or childhood adversity) has recently been associated with altered prefrontal structure, cerebral activity and functional connectivity in the resting state (Cisler et al., 2013; Gupta et al., 2014; Philip et al., 2013b; van der Werff et al., 2013a; van der Werff et al., 2013b), as well as altered limbic/prefrontal brain function during emotion processing (Fonzo et al., 2013). Few studies have investigated the effect of ELS on functional brain activation during noxious visceral stimulation. The current study addresses this in a rodent model of ELS. We also explore sex differences in the effects of ELS on brain functional responses, based on a broad literature demonstrating that women are at greater risk for experiencing many forms of functional pain disorders, including IBS (Berman et al., 2000; Labus et al., 2008; Mayer et al., 2004; Naliboff et al., 2003; Unruh, 1996), as well as a literature suggesting that altered neonatal maternal care may impact adult emotionality and stress responsiveness in a sexually dimorphic manner (Champagne et al., 2008; Champagne and Curley, 2008; Faraday, 2002; Lehmann and Feldon, 2000; Lehmann et al., 1999; Wigger and Neumann, 1999). We hypothesize that ELS-associated visceral hyperalgesia is associated with increased activation in the pain circuit, and that ELS effects on brain functional responses will show sex differences.

2. Materials and methods

2.1. Animals

All experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of the University of Southern California and are in accordance with the guidelines of the Committee for Research and Ethical Issues of the International Association or the Study of Pain. Adult female and male Wistar rats (3 month old) were purchased from Harlan Sprague Dawley (Indianapolis, IN, USA) and were housed in the vivarium on a 12-h light/12-h dark cycle with free access to water and rodent chow. Two weeks after arrival to the vivarium, breeding was initiated in separately housed breeding pairs (1 male, 1 female) for the production of male and female offspring. Four litters were used. To minimize potential inter-litter differences in animal behavior, female pups (#2–3) from each litter were randomized to either an ELS/female or control/female group; males were similarly randomized to an ELS/male or control/male group. The following experimental groups were examined: early life stress female (ELS/female, n = 10) and male rats (ELS/male, n = 12); and no-stress female (CON/female, n = 8) and male controls (CON/male, n = 10).

2.2. Limited bedding model

The limited bedding model was introduced by Gilles et al. (Gilles et al., 1996) as an alternate model of maternal neglect and abuse that, unlike the maternal separation model, allows continuous presence of the mother. Minimal nesting material is provided to the dam following delivery of her pups, resulting in increased maternal anxiety and decreased nurturing behavior (licking/grooming), increased rough handling, and increased pup vocalization (Ivy et al., 2008; Raineki et al., 2012). Long-term consequences on the offspring include increased stress hormone release, reduced expression of hypothalamic corticotrophin releasing hormone, adrenal hypertrophy (Avishai-Eliner et al., 2001), decreased social and exploratory behavior (Ivy et al., 2008; Raineki et al., 2012), increased learned helplessness in the Porsolt swim test (Raineki et al., 2012), impaired visual-spatial memory in the Morris Water maze (Ivy et al., 2010), decreased dendritic branching in the CA1 area of the hippocampus (Ivy et al., 2010), increased corticotropin-releasing hormone-positive interneurons in the hippocampus (Ivy et al., 2010), and increased c-fos expression in the amygdala in response to a stress challenge (Raineki et al., 2012). Methods were as previously reported (Guo et al., 2015). One day after testing, plug-positive dams were individually housed under standard vivarium conditions. The day of birth was termed postnatal day 0. On postnatal day 2, litters and dams were transferred to a standard cage equipped with a steel grid (2.5 cm above the floor, 5 × 5 mm mesh). The only bedding material available was a paper towel (1 paper towel for 8–12 pups, ½ towel for <8 pups) that the dam

would shred and uses to make her nest. The paper towel was replaced at postnatal day 4/5. Control groups were left undisturbed and maintained with standard woodchip bedding. On postnatal day 9 all litters and dams exposed to limited bedding were returned to standard housing conditions using woodchip bedding. Upon weaning on postnatal day 21, offspring were separately placed in same sex, social groups of 2 or 3, and housed until 10–11 weeks of age under standard vivarium conditions.

2.3. Surgical procedures

Animals at age 8–10 weeks were anesthetized (isoflurane 2% in 70% oxygen and 30% nitrous oxide). Rats were implanted with a chronic percutaneous, external jugular vein catheter (5.0 French silastic). In addition, a telemetry transmitter (TA11-CTA-F40, Data Sciences Intl., St. Paul, MN, USA) was implanted subcutaneously on the dorsum of the animal just caudal to the scapula. A skin incision was made on the abdomen and electrodes of the transmitter were tunneled subcutaneously to the abdominal incision. Tips of the electrodes were bared, placed in parallel (0.5 cm apart), and stitched into the left external oblique musculature, just superior to the inguinal ligament. The receiver platform was linked via a data exchange matrix to a computer. Implants could be turned on and off with an external magnet to send a radiofrequency signal of electromyographic (EMG) activity to a receiver platform placed underneath the experimental cage on the day of recording. All animals were allowed to recover for seven days.

2.4. Colorectal distension and injection of the cerebral perfusion tracer

The rats were habituated to an uninflated colorectal balloon and the experiment cage for 45 min per day for three days prior to the CBF mapping using our prior methods (Wang et al., 2009). Briefly, under light isoflurane anesthesia (1.5% isoflurane × 3 min), a flexible latex balloon (length = 6 cm) was inserted intra-anally such that its caudal end was 1 cm proximal to the anus. The tubing connecting the balloon and the barostat (Distender Series II, G&J Electronics Inc., Toronto, Canada) was fixed to the base of the tail with adhesive tape and covered by a stainless steel spring for protection against animal biting. Animals were allowed to recover for 30 min in the experiment cage, the floor of which was covered with bedding from the animal's home cage. At the end of the recovery period, a piece of tubing filled with the radiotracer, [^{14}C]-iodoantipyrine (80 μCi/kg in 300 μL of 0.9% saline, American Radiolabeled Chemicals, St. Louis, MO, USA) was connected to the animal's venous cannula on one end, and to a syringe filled with euthanasia agent (pentobarbital 60 mg/kg, 3 mol/L potassium chloride) on the other. The nonrestrained animal was allowed to rest for 5 min before receiving a continuous single colorectal distension (CRD) of 60-mmHg. Thirty-five seconds after the onset of distension, radiotracer was infused by a motorized pump at 2.25 mL/min, followed immediately by euthanasia, which resulted in cardiac arrest within 8–10 s, termination of brain perfusion, and death. This 8–10 s time window provided the temporal resolution during which the distribution of regional cerebral blood flow related tissue radioactivity (rCBF) was mapped.

2.5. Brain slicing and autoradiography

Brains were rapidly removed, flash frozen in methylbutane/dry ice, and cryosectioned into 20-μm-thick coronal slices (57 slices beginning at 4.7 mm anterior to bregma, inter-slice spacing 300 μm). Slices were exposed for 5 days at room temperature to Kodak Biomax MR film (Sigma–Aldrich, St. Louis, MO, USA), and images were digitized on an 8- bit gray scale using a voltage stabilized light box (Northern Lights Illuminator, Interfocus Imaging Ltd., Cambridge, UK) and a Retiga 4000R charge-coupled device monochrome camera (Qimaging, Surrey, Canada).

2.6. Abdominal EMG analysis

The visceromotor response (VMR) was quantified by measuring EMG activity in the external oblique musculature.

EMG signals were continuously recorded by radiotelemetry at a sampling rate of 1 kHz, digitized and stored on a computer with the Dataquest ART 3.0 software (Data Sciences Intl., St. Paul, MN, USA). EMG waveforms were lowcut filtered at 20 Hz to eliminate movement interference, and then full-wave rectified. Each rat's rectified EMG signal was averaged over consecutive 1-s intervals for the 40-s period before (baseline) and 40-s after the onset of CRD (pain response). Each data point was normalized to average baseline amplitude (defined as 100%). Area under the curve was calculated for the 40-s period after CRD onset.

Group differences in the EMG were separately tested by a two-way ANOVA ($p < 0.05$) using the between-subject factors of 'Sex' (male, female) and 'ELS' (ELS, controls), with post-hoc testing using Fisher's least significant difference. Data were presented as mean ± SEM.

2.7. Statistical parametric mapping

rCBF was quantified by autoradiography and analyzed on a whole-brain basis using statistical parametric mapping (SPM, version SPM5, Wellcome Centre for Neuroimaging, University College London, London, UK). SPM, a software package developed for analysis of imaging data in humans (Friston et al., 1991) which has been adapted by us (Nguyen et al., 2004) for use in autoradiographs of the rat brain. A 3-D reconstruction of each animal's brain was conducted using 57 serial coronal sections. Adjacent sections were aligned both manually and using TurboReg, an automated pixel-based registration algorithm (Thevenaz et al., 1998). A typical reconstructed brain had ~2,750,000 voxels (voxel size: 40 μm × 40 μm x 300 μm). After 3-D reconstruction, one "artifact free" male brain was selected as reference and smoothed with a Gaussian kernel (FWHM = 3 × voxel dimension). Brains from all animals were spatially normalized to the smoothed reference brain. Following spatial normalization, normalized images were averaged to create a mean image, which was then smoothed to create the final brain template. Each original 3-D reconstructed brain was then spatially normalized into the standard space defined by the template. An unbiased, voxel-by-voxel analysis of whole-brain activation using SPM was used for detection of significant changes in functional brain activation between rats exposed to CRD and controls. Global differences in the absolute amount of radiotracer delivered to the brain were adjusted in SPM for each animal by scaling the voxel intensities so that the mean intensity for each brain was the same (proportional scaling). Unbiased, voxel by-voxel, between-group statistical analyses were performed in SPM to identify significant task-related changes in functional brain activation. We first ran a factorial analysis, equivalent to running an ANOVA test at each voxel, to identify rCBF changes reflecting main effects of 'ELS', 'Sex', and 'Sex x ELS' interaction. To further delineate direction of the rCBF changes identified in the factorial analysis, we performed between-group Student's t-test. To control both Type I and Type II errors, we set a significance threshold of $p < 0.05$ for individual voxels within clusters of at least 100 contiguous significant voxels (extent threshold). Brain regions were identified using coronal, sagittal and transverse views according to the rat brain atlas (Paxinos and Watson, 2007). To identify brain regions showing

rCBF correlations with EMG, SPM analyses using EMG (area under the curve) as a covariate was run for all animals pooled together, as well as for pooled ELS animals (ELS/female, ELS/male) and pooled controls (CON/female, CON/male). Significance level for Pearson's correlation coefficient was set at $p < 0.05$ and an extent threshold of 100 contiguous voxels.

2.8. Functional connectivity

We applied a region of interest (ROI) seed correlation analysis to assess functional connectivity of the amygdala (lateral/basolateral nucleus), as well as the locus coeruleus/lateral parabrachial nucleus. Structural ROIs were hand drawn in MRIcro (version 1.40, http://cnl.web.arizona.edu/mricro.htm) over the left hemisphere of the template brain according the rat brain atlas. The structural ROIs were then intersected with significant clusters ($p < 0.05$ for >100 contiguous voxels) defining regional functional activation showing the main effects of the factorial analyses to create functional seed ROIs. Mean optical density of the seed ROIs were extracted for each animal using the MarsBaR toolbox for SPM (version 0.42, http://marsbar.sourceforge.net/) (Brett et al., June, 2002). Correlation analysis was performed in SPM for each group, as well as for pooled ELS animals (ELS/female, ELS/male) and pooled controls (CON/female, CON/male), using the seed values as a covariate. Threshold for significance was set at $p < 0.05$ at the voxel level and an extent threshold of 100 contiguous voxels. Regions showing significant correlations (positive or negative) in rCBF with the seed ROI were considered functionally connected with the seed.

In addition, we calculated significant inter-regional correlations as a measure of functional connectivity and compared degree centrality for brain regions within the pain circuit, as well as associated limbic/paralimbic areas (Fig. 5). Degree centrality was defined as the number of significant correlations (positive or negative, $p < 0.05$) linking an individual ROI (extracted as noted above) to the other ROIs. ROIs included were the anterior and posterior insula, prelimbic (prefrontal), primary somatosensory, ventral cingulate, and retrosplenial cortices, anterior hypothalamus, periaqueductal gray, hippocampus (CA1), median raphe, lateral septum, lateral/basolateral amygdala, central amygdala, thalamus (mediodorsal, posterior, central medial, ventral-posterolateral/ventral-posteromedial nuclei), bed nucleus of the stria terminalis, locus coeruleus/lateral parabrachial nucleus, external globus pallidus, and ventral tegmental area (parabrachial pigmented nucleus). For the ELS animals (ELS/female, ELS/male) and the controls (CON/female, CON/male), the degree centrality for each ROI was calculated. Group differences were displayed using a bar graph, i.e. the degree centrality of ELS animals (ELS/female, ELS/male) minus that of controls animals (CON/female, CON/male). This allowed for intuitive visualization of the effect of ELS on the degree metrics, with differences in degree interpreted in a qualitative manner.

3. Results

3.1. ELS elicits visceral hyperalgesia in adult rats

ANOVA revealed a significant main effect of 'ELS' ($F_{1,36} = 10.48$, $p = 0.003$) on the VMR response to 60-mmHg of CRD (Fig. 1). The main effect of 'Sex' and the 'Sex x ELS' interaction were not significant. Post-hoc tests (Fisher's least significant difference) revealed significant pairwise differences for ELS/male vs. CON/male ($p = 0.04$) and ELS/female vs. CON/female ($p = 0.02$), but not ELS/female vs. ELS/male ($p = 0.56$) or CON/female vs. CON/male ($p = 0.82$).

3.2. Effects of ELS on regional functional brain activation

During acute noxious visceral stimulation, factorial analysis revealed a significant main effect of 'ELS' broadly across cerebral cortex, including the ventral cingulate (Cg2), prelimbic (PrL), retrosplenial (RS), posterior insular (pIns); primary somatosensory (forelimb, S1FL; hindlimb, S1HL; upper lip, S1ULp; barrel field, S1BF), and to a lesser extent, anterior insular (aIns), ectorhinal/temporal association (Ect/TeA), secondary somatosensory (S2), and primary and secondary visual (V1, V2) cortices (Fig. 2, column 3; Table 1, $p < 0.05$, clusters of >100 contiguous voxels).

Subcortical areas showing main 'ELS' effect included the amygdala (central n., Ce; lateral n./basolateral n., La/BL), hippocampus (dentate gyrus, DG; CA1), thalamus (ventral −posterolateral n./ventral posteromedial n., VPL/VPM; mediodorsal n., MD; central medial n., CM; posterior n., Po; paraventricular n. posterior, PVP; reticular n., Rt; habenula n., Hb; medial geniculate n., MG), anterior pretectal n. dorsal (APTD), periaqueductal gray (PAG), locus coeruleus/lateral parabrachial area (LC/LPB), parabrachial pigmented nucleus of the ventral tegmental area (PBP), lateral septal nucleus (LSI, intermediate part), external globus pallidus (GPe), superior colliculus (SC) and cerebellum (simple lobule, Sim; vermis, 1Cb, 3Cb, 5Cb). Significant, though less broadly represented, were changes in the median raphe n. (MnR) and hypothalamus (anterior area, AH; lateral peduncular, PLH).

Significant rCBF changes showing a main effect of 'ELS' were further evaluated using between-group Student's t-tests. In males (Fig. 2, column 1; Table 1), ELS compared to controls showed significantly increased rCBF in the cingulate (Cg2, dorsal, Cg1), PrL, secondary motor (M2), RS, V1 and V2 cortices ($p < 0.05$, clusters of >100 contiguous voxels). Subcortically, significantly greater rCBF was noted in the PAG, caudate-putamen (CPu, ventral and lateral), thalamus (Hb, MG), APTD, PBP, LSI, hypothalamus (AH, VMH, PLH), hippocampus (CA1 and posterior DG), and SC. Significant decreases in rCBF were noted in the insula (aIns, pIns), orbital (ventral, VO; lateral, LO), and primary and secondary somatosensory cortex (S1BF, S1ULp, left S1HL, S1FL, S2), as well as in the amygdala (La/BL, Ce), thalamus (VPL/VPM, MD, PVP, Po), and cerebellum (Sim, vermis).

Female ELS animals compared to controls (Fig. 2, column 2; Table 1), similar to findings in males, showed significantly increased rCBF in the Cg2, PrL, RS, V1 and V2 cortices, as well as

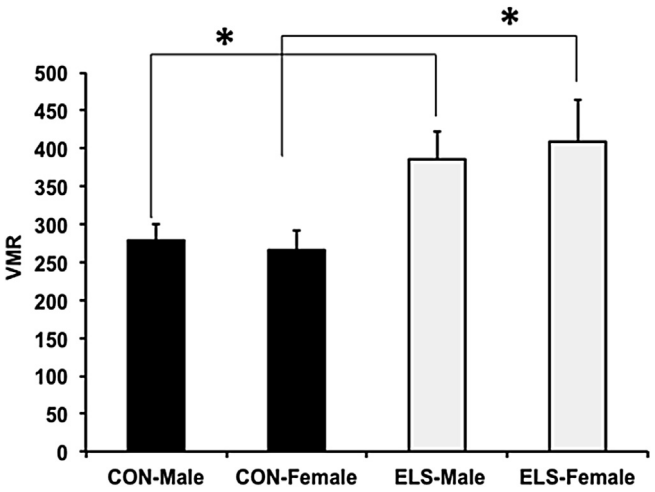

Fig. 1. Effect of ELS on visceromotor response (VMR) to colorectal distension (60-mm Hg) in adult rats. VMR (mean ± standard error) is shown for stressed female (n = 10), control female (n = 8), stressed male (n = 12), and control male rats (n = 10). *: $p < 0.05$.

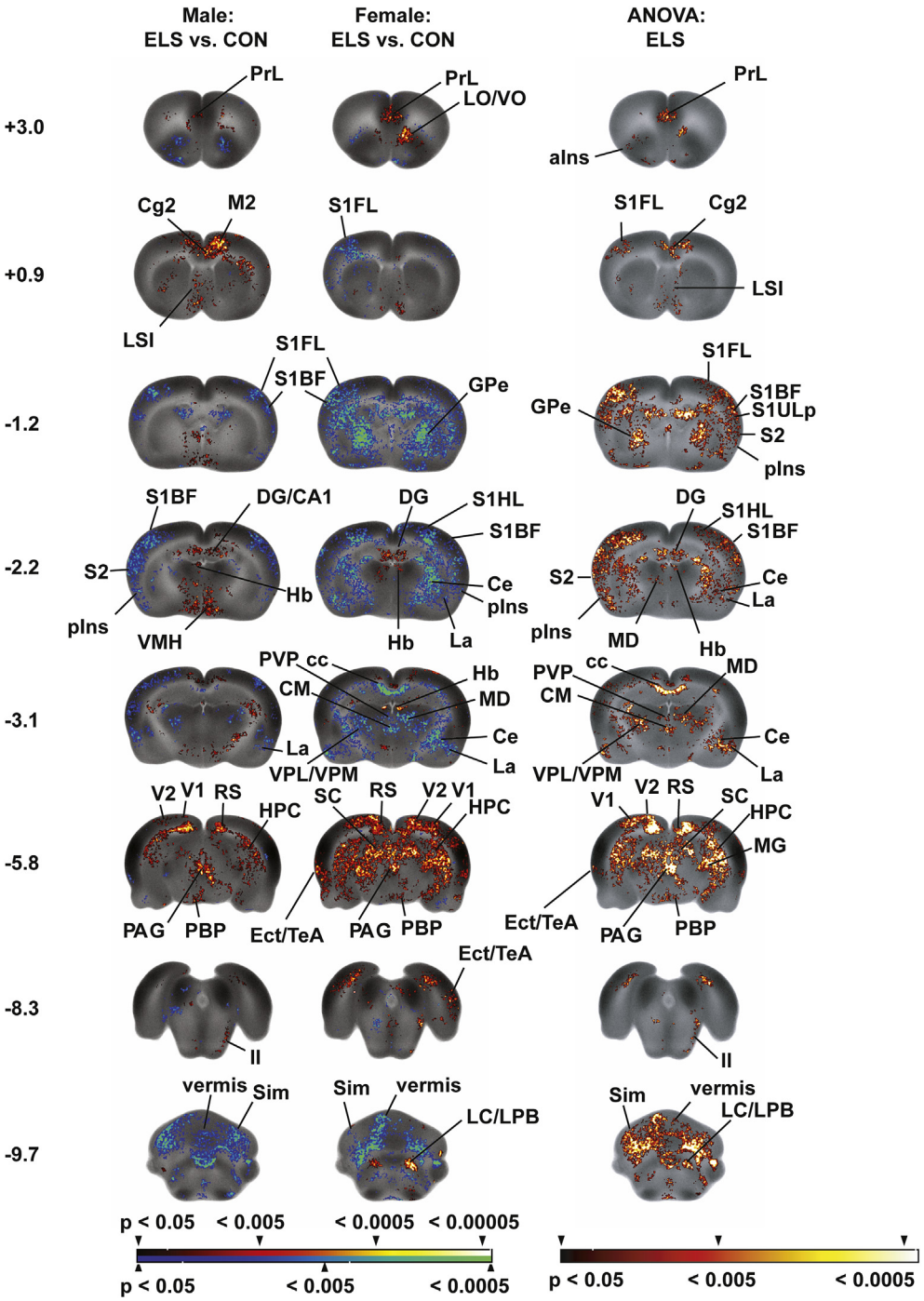

Fig. 2. Significant effect of stress on regional cerebral blood flow (rCBF) during noxious visceral stimulation shown as color coded overlays on a selection of representative coronal slices of the template brain. Significant changes in rCBF comparing stress and controls in males (ELS/male vs. CON/male) and females (ELS/female vs. CON/female) are respectively shown in columns 1 and 2 (Student's t test, red: increase in rCBF, blue: decrease in rCBF) with anterior-posterior locations relative to bregma (mm) denoted on the left. Results of the main effect of 'ELS' by factorial analysis are shown in column 3. Abbreviations: Acb (Accumbens n.), aIns (anterior insula), cc (corpus callosum), La (lateral amygdala), CA1 (CA1 field of hippocampus), Ce (amygdala, central n.), Cg1, Cg2 (cingulate cortex area 1 dorsal, area 2 ventral), CM (central medial thalamic n.), CPu (dorsal caudate-putamen), DG (dentate gyrus), dHPC (dorsal hippocampus), Ect/TeA (ectorhinal/temporal association cortex), GPe (globus pallidus, external), Hb (habenula), HPC (hippocampus), IC (inferior colliculus), La (amygdala, lateral n.), LC/LPB (locus coeruleus/lateral parabrachial n.), ll (lateral lemniscus), LD (lateral dorsal thalamic n.), LO/VO (lateral/ventral orbital cortex), LSI (lateral septum intermediate), M1/M2 (primary/secondary motor cortex), MD (mediodorsal thalamic nucleus), Me (amygdala, medial n.), MG (medial geniculate), MnR (median raphe n.), MPA (hypothalamic medial preoptic area), PAG (periaqueductal gray), PBP (parabrachial pigmented n. of the ventral tegmental area), pIns (posterior insula), pIns (posterior insula), pIns/Ect (posterior insula/ectorhinal cortex transition area), Po (posterior thalamic n.), PrL (prelimbic cortex), PtA (parietal association cortex), PVP (posterior paraventricular thalamic n.), RS (retrosplenial cortex, 'posterior cingulate'), S1BF/S1FL/S1HL/S1J/S1ULp (primary somatosensory cortex, barrel field/forelimb/hindlimb/jaw/upper lip area), S2 (secondary somatosensory cortex), SC (superior colliculus), Sim (simple cerebellar lobule), STM (bed n. of the stria terminalis), V1/V2 (primary/secondary visual cortex), VMH (ventromedial hypothalamus), VPL/VPM (ventral-posterolateral/ventral-postereromedial thalamic n.). The right side of the images corresponds to the left side of the brain.

Table 1
Effects of ELS on functional brain activation during acute colorectal distension for regions within pain and limbic/paralimbic circuits. Significant changes in rCBF comparing stress effects in males (ELS/male vs. CON/male) and in females (ELS/female vs. CON/female) are respectively shown in columns 1 and 2 (Student's t test). Shown are increases ('+') and decreases ('−') in cerebral blood flow tracer distribution in the right/left hemispheres, with symbols in parentheses denoting changes less broadly represented, and 'a' denoting 'anterior' and 'p' denoting 'posterior' regions. Column 3 shows significance changes ('*') of the main effect of 'ELS' from the ANOVA. Significances are shown at the voxel level ($p < 0.05$) for clusters of >100 contiguous voxels. Abbreviations are taken from the Paxinos and Watson rat brain atlas (Paxinos and Watson, 2007).

	Male: ELS vs. CON	Female: ELS vs. CON	ELS
Cortex			
Cingulate, dorsal (Cg1)	(+)/		
Cingulate, ventral (Cg2)	+/+	(+)/(+)	*/*
Ectorhinal/temporal association area (Ect/TeA)		+/+	/*
Insula, anterior (aIns)	(−)/	(−)/	(*)/
Posterior (pIns)	−/(−)	(−)/−	*/*
Motor, secondary (M2)	+/+		/*
Orbital, lateral/ventral (LO/VO)	−/−	(+)/+	(*)/
Prelimbic (PrL)	+/	+/+	*/*
Retrosplenial, granular (RS)	+/+	+/+	*/*
Somatosensory, primary, barrel field (S1BF)	−/−	−/−	*/*
Forelimb (S1FL)	−/−	−/−	*/*
Upper lip (S1ULp)	−/−	−/−	*/*
Hindlimb (S1HL)	/(−)	−/−	/*
Somatosensory, secondary (S2)	−/−	−/−	*/*
Visual, primary, secondary (V1, V2)	(+)/(+)	+/+	*/*
Subcortex			
Amygdala, central n. (Ce)	/(−)	−/−	(*)/*
Lateral/basolateral (La/BL)	/−	−/−	(*)/*
Medial (Me)			
Bed n. of the stria terminalis (STM)			
Caudate-putamen (CPu), mediodorsal			
Lateral	/+	−/	
Ventral	(+)/(+)	+/+	(*)/(*)
Cerebellar simple lobule (Sim)	−/−	(+)/+	*/*
Vermis, lobules 1, 3, 5 (1Cb, 3Cb, 5Cb)	−	−	*
Colliculus, inferior (IC)		/(+)	
Superior (SC)	(+)/(+)	+/+	*/*
Globus pallidus, external (GPe)		−/−	*/*
Hippocampus, dentate gyrus (DG, anterior, posterior)	(+)/+ p	+/+	*/*
CA1 (anterior, posterior)	(+)/+	+/+p	*/*
Hypothalamus, anterior (AH)	+/+	+/(+)	(*)/(*)
Ventromedial (VMH)	(+)/+		
Lateral peduncular (PLH)	(+)/	−/−	(*)/(*)
Locus coeruleus/lateral parabrachial n. (LC/LPB)		+/+	*/*
Parabrachial pigm. n./ventral tegmental area (PBP)	+/+	+/+	*/*
Periaqueductal gray (PAG)	+	+	*
Pretectal n., anterior-dorsal (APTD)	(+)/(+)	+/+	*/*
Raphe, median (MnR)		+	(*)
Septum, lateral n., intermediate part (LSI)	+/+	+/+	*/*
Thalamus, central medial n. (CM)		−/−	*
Habenular n. (Hb)	+/(+)	+/+	*/*
Mediodorsal n. (MD)	(−)/	−/−	*/*
Medial geniculate (MG)	/+	+/+	*/*
Paraventricular, posterior n. (PVP)	(−)	−	*
Posterior n. (Po)	−/	−/−	*/*
Reticular n. (Rt)		−/(−)	*/(*)
Ventral-posterolateral/ventral-posteromedial n. (VPL/VPM)	−/	−/−	*/*

subcortically in the PAG, ventral CPu, the thalamus (Hb, MG), APTD, PBP, LSI, hypothalamus (AH), hippocampus (DG, posterior CA1) and SC ($p < 0.05$, clusters of >100 contiguous voxels). Likewise, significant decreases in cortical rCBF were noted in the insula (aIns, pIns), primary and secondary somatosensory cortex (S1BF, S1ULp, S1HL, S1FL, S2), and in subcortical rCBF in the amygdala (La/BL, Ce), thalamus (VPL/VPM, MD, CM, PVP, Po) and cerebellar vermis. These decreases paralleled those in males, as noted above.

3.3. Correlation of EMG with rCBF

When all animals were pooled together, EMG responses showed significant, positive correlation with rCBF in the amygdala and retrosplenial cortex, and significant, negative correlation in the prefrontal cortex (PrL; infralimbic, IL; Cg1), as well as in primary somatosensory cortex and posterior insular cortex ($p < 0.05$, clusters of >100 contiguous voxels). EMG correlation in pooled ELS animals (female, male) compared to that in pooled controls (female, male) showed a substantially broader positive correlation with rCBF in the amygdala, as well as less broad negative correlation in the prefrontal areas (Cg1, PrL) (Fig. 3).

3.4. Effect of ELS on functional connectivity of the amygdala

Seed analysis for the amygdala (La/BL) revealed that ELS animals (female, male) compared to controls (female, male) showed greater significant positive functional connectivity to the PAG, somatosensory cortices (S1J, S2), insula (aIns, pIns), nucleus accumbens (Acb), CPu (mediodorsal, ventral), bed nucleus of the stria terminalis (STM), and hypothalamus (AH; PLH; VMH; medial preoptic area, MPA) (Fig. 4). ELS animals compared to controls also showed greater significant negative functional connectivity to the RS and MnR, while controls showed greater negative functional connectivity to Cg1, as well as primary and secondary motor cortices (M1,

M2).

3.5. Effect of ELS on degree centrality

ELS animals compared to controls showed a relative increase in degree centrality across several regions of the pain circuit, including the insula (aIns, pIns), amygdala (La/BL), LC/LPB, RS, ventral cingulate, as well as the bed nucleus of the stria terminalis, lateral septum, and anterior hypothalamus. Decreases were noted particularly in the thalamus (VPL/VPM) and hippocampus (CA1 region) (Fig. 5).

3.6. Effects of 'Sex' on regional functional brain activation

During acute noxious visceral stimulation, factorial analysis revealed main effect of 'Sex' in the following cortical regions (Fig. 6, column 3; Table 2): Cg1, Cg2, PrL, RS, Ect/TeA, M2, primary somatosensory cortex (S1FL, S1ULp), V1 and V2 ($p < 0.05$, clusters of >100 contiguous voxels). Subcortically significant sex differences were noted in the amygdala (Ce; La/BL; medial, Me), hippocampus (anterior CA1, anterior DG), MnR, LSI, PAG, CPu (mediodorsal, lateral, ventral), and thalamus (CM, Hb, MD, Po, Rt, VPL/VPM).

Significant rCBF changes showing a main 'Sex' effect were further evaluated using between-group Student's t-tests. Female ELS compared to male ELS animals (Fig. 6, column 1; Table 2) showed significantly greater rCBF in RS, limited regions of primary somatosensory cortex (S1ULp), and subcortically in the amygdala (Me, Ce), hippocampus (DG), thalamus (Hb, Rt), LC/LPB, MnR, CPu (lateral), and cerebellum (Sim, vermis). Significant decreases in rCBF were noted in cingulate (Cg1, Cg2), aIns, Ect/TeA, M2, and primary somatosensory cortex (S1HL, S1FL), and subcortically in the STM, LSI, PAG, CPu (mediodorsal, ventral), hypothalamus (AH, VMH, PLH), and thalamus (CM, MD, Po, VPL/VPM).

3.7. Effects of 'Sex x ELS' interaction on regional functional brain activation

Significant 'Sex x ELS' interaction (Fig. 6, column 4; Table 2) was noted in the Cg1, pIns, orbital cortex (LO/VO), Ect/TeA, M2, primary somatosensory cortex (S1FL), V1 and V2 ($p < 0.05$, clusters of >100 contiguous voxels). Subcortically, significant 'Sex x ELS' interaction was noted in the LC/LPB, amygdala (La, Ce), STM, CPu (lateral, ventral), GPe, cerebellum (Sim; vermis, 5Cb), hippocampus (anterior CA1), hypothalamus (AH, VMH, PLH), APTD, STM, thalamus (CM, Hb, MG, Rt, VPL/VPM), and the colliculi (superior, SC; inferior, IC).

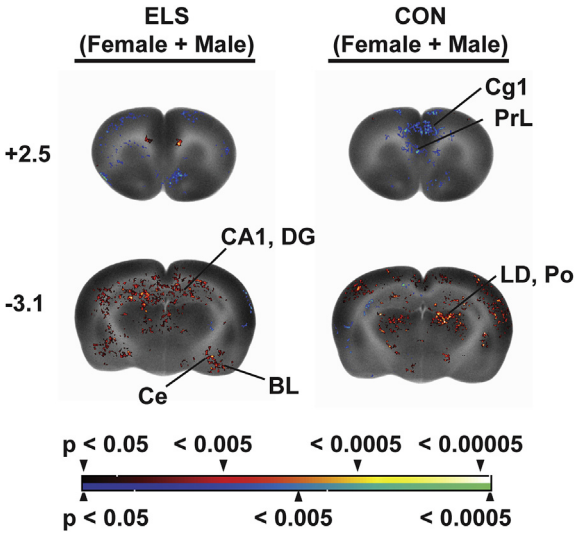

Fig. 3. Correlation of EMG activity with rCBF. Significant correlation is shown for the amygdalar and prefrontal region of stress animals (ELS/female, ELS/male) and controls (CON/female, CON/male). Color coded overlays show regions significantly correlated (red: positive correlation, blue: negative correlation, $p < 0.05$, cluster >100 voxels). Abbreviations: see legend Fig. 2. The right side of the images corresponds to the left side of the brain.

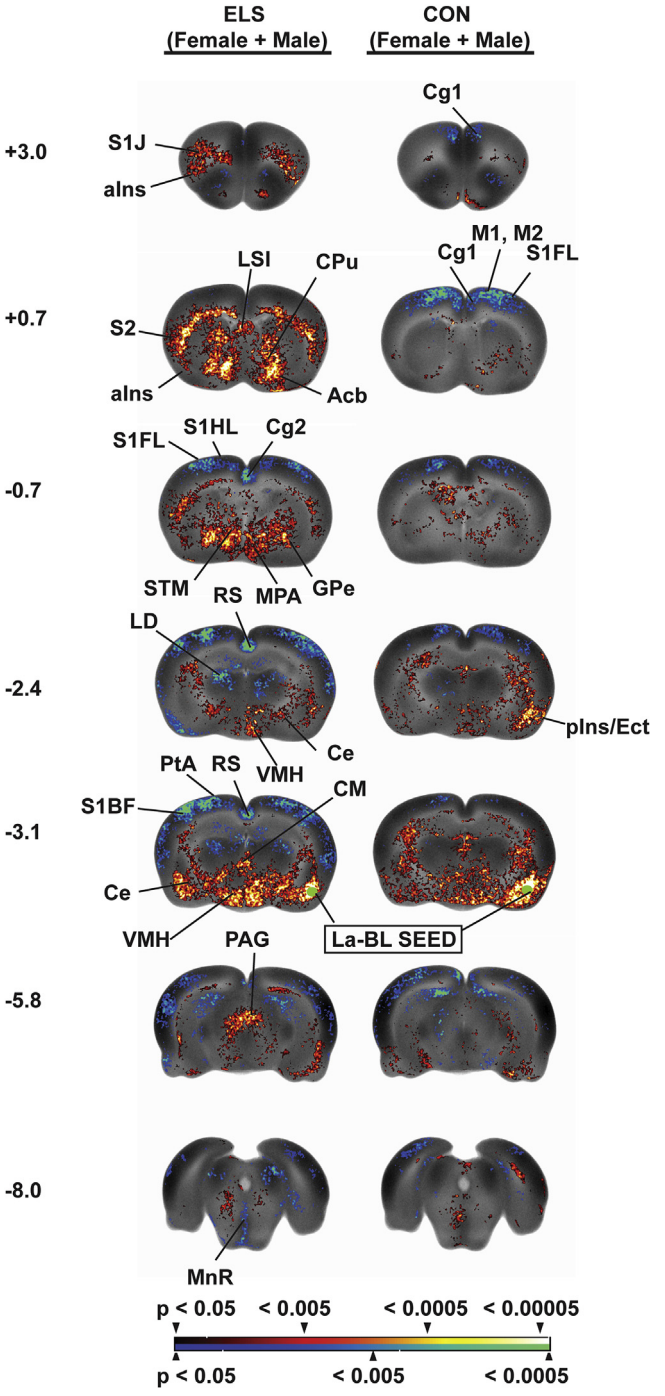

Fig. 4. ELS alters functional connectivity of the amygdala. Color coded overlays show brain regions significantly correlated with a left lateral/basolateral amygdala seed (red: positive correlation, blue: negative correlation). Comparison of the functional correlation across the brains of ELS animals (ELS/female, ELS/male) and controls (CON/female, CON/male). Abbreviations: see legend Fig. 2. The right side of the images corresponds to the left side of the brain.

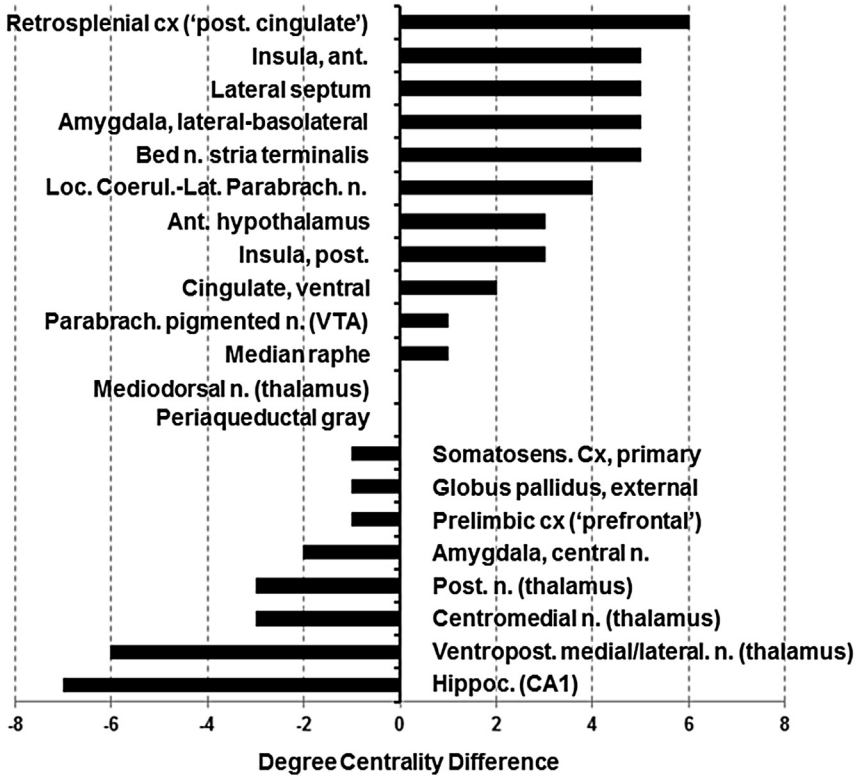

Fig. 5. Effects of ELS on degree centrality within the pain and stress networks. Shown are group differences in the total number of significant interregional correlations (positive or negative) linking an individual region-of-interest (ROI) to all other ROIs ('degree centrality'). Degree centrality of control animals (CON/female, CON/male) are subtracted from that of ELS animals (ELS/female, ELS/male). Abbreviations: see legend Fig. 2.

Different from males, female ELS animals compared to female controls, showed increased rCBF in the orbital cortex (LO/VO), Ect/TeA, as well as subcortically in the LC/LPB, MnR, and lateral cerebellum (Sim) (Fig. 2, Table 1). rCBF was decreased in female ELS animals compared to female controls (different from males) in the CPu (lateral), GPe, and thalamus (CM, Rt). Unlike males, no significant increases in rCBF were seen in the hypothalamus in females.

Sex differences in ELS animals differed from those noted in controls. In ELS animals, females compared to males showed a lower or unchanged rCBF in PrL and primary somatosensory cortex (S1ULp), amygdala (La/BL), ventral CPu, GPe, anterior CA1 region of the hippocampus, and thalamus (VPL/VPM, CM). In control animals, however, these sex differences showed a relative increase in rCBF (Fig. 6, Table 2). Increased significant sex differences in rCBF of ELS animals (compared to decreases in controls) were noted in the LC/LPB, and cerebellum (Sim, vermis) ($p < 0.05$, clusters of >100 contiguous voxels). A seed analysis for the LC/LPB revealed significant functional connectivity with broad areas of the amygdala (Ce, Me, BL, La, contralateral > ipsilateral) in ELS females, whereas functional connectivity between the LC/LPB and amygdala was negative in ELS males and control females, and absent in control males (Fig. 7).

4. Discussion

4.1. Visceral hyperalgesia

Our study demonstrated the presence of visceral hyperalgesia in adult animals with a prior history of ELS. These results independently confirmed our earlier EMG findings in the limited bedding model (Guo et al., 2015), and those of others (Prusator and Greenwood-Van Meerveld, 2015), as well as similar findings reported for the maternal separation rat model (Bian et al., 2010; Coutinho et al., 2002; Welting et al., 2005). Similar to our earlier results, we did not see a sex difference in the VMR response of stressed animals. The current study did not show a lower visceral pain threshold in female controls compared to male controls, we had noted in our earlier study (Guo et al., 2015). The animals in the current study had a single exposure to 60-mm Hg CRD, whereas the earlier study involved a titrated sequence of multiple CRDs that preceded the 60-mm Hg distension. It is possible that these methodologic differences may have shaped the final VMR to 60-mm Hg of noxious visceral stimulation.

4.2. Effects of ELS on regional functional brain activation

In animal models, ELS has been reported to increase functional activation in the amygdala during an acute stress challenge, though depending on the paradigm, changes have also been reported in the hippocampus, insula, and paraventricular hypothalamic nucleus (Brydges et al., 2013; Raineki et al., 2012; Sadler et al., 2011; Sanders and Anticevic, 2007; Troakes and Ingram, 2009; Tsuda and Ogawa, 2012). Few studies have examined the effects ELS has on functional brain responses elicited during acute pain. Wouters et al. using $H_2[O]^{15}$-micro-positron emission tomography in anesthetized rats showed that visceromotor responses in rats with a history of neonatal maternal separation were associated with activation of the periaqueductal gray, hippocampus, and primary somatosensory cortex, as well as deactivation of the frontal cortex, though no comparison with a normally reared control group was made (Wouters et al., 2012). Chung et al. demonstrated that rats with a history of maternal separation showed greater c-fos expression during acute CRD than control animals in the cingulate cortex, amygdaloid central nucleus, and paraventricular thalamic nucleus,

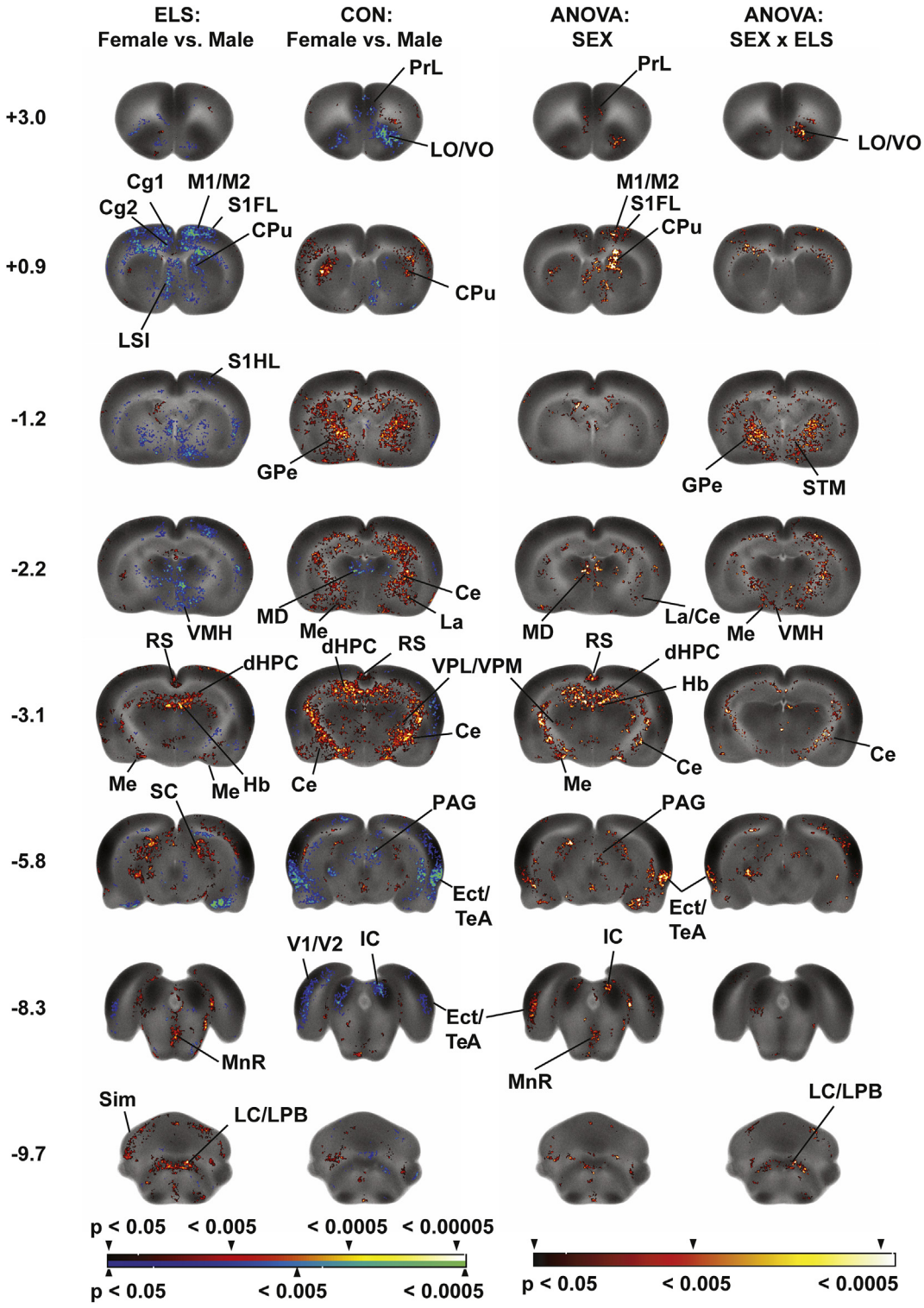

Fig. 6. Significant effect of sex on regional cerebral blood flow (rCBF) during noxious visceral stimulation shown as color coded overlays on a selection of representative coronal slices of the template brain. Significant changes in rCBF comparing females and males with stress (ELS/female vs. ELS/male) and without stress (CON/female vs. CON/male) are respectively shown in columns 1 and 2 (Student's t test, red: increase in rCBF, blue: decrease in rCBF). Results of the main effect of 'Sex' and 'Sex x ELS' interaction by factorial analysis are shown respectively in columns 3 and 4. Abbreviations: see legend Fig. 2. The right side of the images corresponds to the left side of the brain.

as well as the lumbosacral spinal cord (Chung et al., 2007). Our study using a whole brain analysis extended these results in the limited bedding model, showing that ELS compared to standard rearing elicits alteration in functional brain responses to CRD broadly across circuits involved in the regulation of pain and emotion as described below.

ANOVA revealed a significant main effect of 'ELS' widely across regions implicated in pain processing (Bromm, 2004; Bushnell

Table 2
Effects of sex on functional brain activation during acute colorectal distension for regions within pain and limbic/paralimbic circuits. Significant changes in rCBF comparing females and males with stress (ELS/female vs. ELS/male) and without stress (CON/female vs. CON/male) are respectively shown in columns 1 and 2 (Student's t test). Shown are increases ('+') and decreases ('−') in cerebral blood flow tracer distribution in the right/left hemispheres, with symbols in parentheses denoting changes less broadly represented, and 'a' denoting 'anterior' and 'p' denoting 'posterior' regions. Columns 3 and 4 respectively show the significance changes ('*') of the main effect of 'Sex' and 'Sex x ELS' interaction from the ANOVA. Significances are shown at the voxel level ($p < 0.05$) for clusters of >100 contiguous voxels. Abbreviations are taken from the Paxinos and Watson rat brain atlas (Paxinos and Watson, 2007).

	ELS: Female vs. Male	CON: Female vs. Male	Sex	Sex x ELS
Cortex				
Cingulate, dorsal (Cg1)	−/−		(*)/	(*)/(*)
Cingulate, ventral (Cg2)	(−)/(−)	(−)/(−)	(*)/(*)	
Ectorhinal/temporal association area (Ect/TeA)	(−)/(−)	−/−	*/*	*/*
Insula, anterior (aIns)	/(−)	/(−)		
Posterior (pIns)	(−)/	(+)/		(*)/
Motor, secondary (M2)	−/−	(−)/(−)	*/*	(*)/*
Orbital, lateral/ventral (LO/VO)		(−)/−		*/*
Prelimbic (PrL)		+/+	*/*	
Retrosplenial, granular (RS)	+/+	+/+	*/*	
Somatosensory, primary, barrel field (S1BF)				
Forelimb (S1FL)	−/−		(*)/(*)	*/(*)
Upper lip (S1ULp)	(+)/(+)	+/+	*/*	(*)/(*)
Hindlimb (S1HL)	(−)/−			
Somatosensory, secondary (S2)				
Visual, primary, secondary (V1, V2)		−/−		(*)/(*)
Subcortex				
Amygdala, central n. (Ce)	(+)/	+/+	*/*	/(*)
Lateral/basolateral (La/BL)		+/+	/*	(*)/(*) La
Medial (Me)	+/+	+/+	*/*	
Bed n. of the stria terminalis (STM)	(−)/−			(*)/*
Caudate-putamen (CPu), mediodorsal	(−)/-	(−)/(−)	(*)/*	
Lateral	(+)/	+/+	*/(*)	(*)/(*)
Ventral	(−)/(−)	(+)/(+)	/(*)	*/*
Cerebellar simple lobule (Sim)	+/(+)	(−)/−		*/*
Vermis (1Cb, 3Cb, 5Cb)	(+) (5Cb)	−		* (5Cb)
Colliculus, inferior (IC)		−/−	*/*	(*)/(*)
Superior (SC)	(+)/+			(*)/(*)
Globus pallidus, external (GPe)		+/+		*/*
Hippocampus, dentate gyrus (DG)	(+)/(+)	+/+	*/* a	
CA1 (anterior, posterior)		(+)/(+) a	*/* a	(*)/a
Hypothalamus, anterior (AH)	(−)/−	(+)/		(*)/(*)
Ventromedial (VMH)	(−)/(−)	(+)/(+)		(*)/(*)
Lateral peduncular (PLH)	(−)/−			*/*
Locus coeruleus/lateral parabrachial n. (LC/LPB)	+/+	(−)/(−)		*/*
Parabrachial pigm. n./ventral tegmental area (PBP)				
Periaqueductal gray (PAG)	(−)	(−)	(*)	
Pretectal n., anterior-dorsal (APTD)		(−)/-		(*)/(*)
Raphe, median (MnR)	+	(+)	*	
Septum, lateral n., intermediate part (LSI)	−/−		*/*	*/
Thalamus, central medial n. (CM)	(−)	+	(*)	(*)
Habenular n. (Hb)	+/+	(+)/(+)	*/*	*/*
Medial geniculate (MG)		(−)/		(*)/
Mediodorsal n. (MD)	−/−	(−)/(−)	*/*	
Paraventricular, posterior n. (PVP)				
Posterior n. (Po)	/(−)	/(−)	/(*)	
Reticular n. (Rt)	/(+)	(+)/+	(*)/*	/(*)
Ventral-posterolateral/ventral-posteromedial n. (VPL/VPM)	(−)/(−)	(+)/(+)	(*)/	(*)/*

et al., 2013; Freund et al., 2010; Hanamori et al., 1998; Krushel and van der Kooy, 1988; Nielsen et al., 2005; Saper, 1982). These included the anterior-ventral cingulate, retrosplenial ('posterior cingulate'), prelimbic ('prefrontal'), somatosensory (primary, secondary), and insular (anterior, posterior) cortices (Hanamori et al., 1998; Krushel and van der Kooy, 1988; Saper, 1982), as well as the sensory thalamus, amygdala, periaqueductal gray, locus coeruleus/lateral parabrachial nucleus, and anterior-dorsal pretectal area (Fig. 2, column 3). In these regions, ELS-related increases in rCBF were noted for both sexes in the anterior-ventral cingulate, retrosplenial, prelimbic cortices and the periaqueductal gray, with decreases in rCBF in the somatosensory cortex, insula, amygdala, and sensory thalamus. Changes in these areas are consistent with anatomical and electrophysiological studies that show possible afferent nociceptive connectivity to these regions (reviewed in (Bushnell et al., 2013; Moloney et al., 2015)).

A significant main effect of 'ELS' was also noted in related limbic/paralimbic regions, including the hippocampus, median raphe nucleus, lateral septum, thalamus (habenula, reticular n.), hypothalamus, parabrachial pigmented nucleus of the ventral tegmental area, retrosplenial cortex, the ventral caudate-putamen, as well as in the cerebellum (Fig. 2, column 3). Of these regions, ELS-related increases in rCBF were noted for both sexes in the hippocampus, habenula, lateral septum, and parabrachial pigmented nucleus/ventral tegmental area, with decreases in rCBF in the cerebellar vermis.

Our findings in the rat CRD model strikingly paralleled prior studies in human subjects that evaluated the effects of ELS on functional brain responses elicited during an emotional challenge. In humans, ELS has been associated with altered responses during audio and/or visual elicited emotional experiences in a large proportion of the brain regions implicated in the current rodent study,

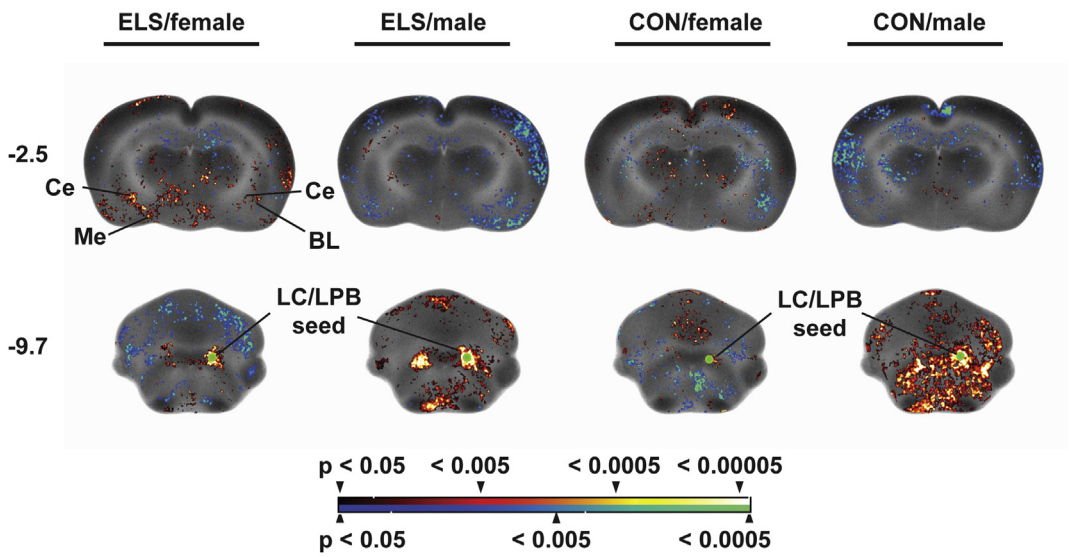

Fig. 7. Functional connectivity of the locus coeruleus/lateral parabrachial complex (LC/LPB). Comparison of the functional connectivity of the LC/LPB in stress-females (ELS/female), stress-males (ELS/male), control-females (CON/female), and control-males (CON/male). Color coded overlays show regions significantly correlated (red: positive correlation, blue: negative correlation) with a left LC/LPB seed. Abbreviations: see legend Fig. 2. The right side of the images corresponds to the left side of the brain.

including the amygdala, insula, cingulate, prefrontal cortex, hippocampus and cerebellum (Aust et al., 2014; Elsey et al., 2015; Fonzo et al., 2013; Yang et al., 2014). Others using animal models have also reported ELS-associated structural alterations in a number of these structures, namely the hippocampus (Frodl and O'Keane, 2013), amygdala (Pechtel et al., 2014; Whittle et al., 2013) and hypothalamus (Kuhlmann et al., 2013), cingulate and insula (Baker et al., 2013; Teicher et al., 2014), with vulnerability of structures dependent in part on the timing of the stressor during neurodevelopment (Bock et al., 2014).

A point of note in our study was that ELS compared to CON resulted in a decrease in regional activation of the amygdala (Fig. 2, rows 3–4). While numerous studies have shown increased amygdala activity during processing of fear-related stimuli, several functional imaging studies have described a relative decrease of amygdala activity during painful stimulation (e.g. (Becerra et al., 2001, 1999; Derbyshire et al., 1997; Naliboff et al., 2006; Petrovic et al., 1999)). It is well known that subjects during voluntary control of their emotional state can modulate amygdala activity (Beauregard et al., 2001; Ochsner et al., 2002; Schaefer et al., 2002). Work by Petrovic et al. has shown that simple contextual manipulation immediately preceding a painful stimulation, that increases the anticipation of the painful event, leads to a decrease in amygdalar activity and modulates the autonomic response during the noxious stimulation (Petrovic et al., 2004). Contextual modulation during aversive stimulation has also been shown to alter responses of the medial prefrontal cortex and insula (Hsieh et al., 1999; Petrovic et al., 2004; Simpson et al., 2001). These observations suggest that the different amygdalar, insular and prefrontal cortical responses noted in ELS compared to CON rats may reflect underlying differences in cognitive control of the visceral pain response.

4.3. *Effects of ELS on functional brain connectivity*

Our study has been the first to examine the effects of ELS brain functional connectivity (FC) during noxious visceral stimulation. Based on the prominent role the amygdala plays in mediating the effects of stress, including ELS (Brydges et al., 2013; Fan et al., 2014; Fonzo et al., 2013; Grant et al., 2011; Pechtel et al., 2014; Suzuki et al., 2014; Whittle et al., 2013; Yang et al., 2014), as well as our finding of a broader positive correlation of the visceromotor response with rCBF in the amygdala of ELS rats compared to controls (Fig. 3, column 1, AP -3.1 mm), we undertook a seed correlation analysis for the amygdala. Results showed that functional correlations with the amygdala were more broadly expressed in ELS animals than in the controls (Fig. 4). In particular, ELS rats demonstrated a significantly more positive FC across the anterior insula, somatosensory cortical regions, as well as with the periaqueductal gray, nucleus accumbens, ventral caudate-putamen and hypothalamus (AH, PLH, VMH, MPA). During CRD, controls showed a negative FC of the amygdala with the anterior-dorsal cingulate (Fig. column 2, AP +3.0 mm, +0.7 mm). ELS rats, however, showed a shift of this negative FC of the amygdala with the cingulate to a more posterior location (retrosplenial cortex, 'posterior cingulate', AP -0.7 mm, -2.4 mm, -3.1 mm). These findings suggest that ELS results in a functional reorganization of the amygdala-driven feedforward inhibition of the medial prefrontal cortex (Ji et al., 2010). ELS elicits a functional disconnection of the amygdala from anterior cingulate regions, while shifting its functional connections to the posterior cingulate that normally show preferential functional connections with somatosensory, rather than limbic areas (Holschneider et al., 2014). ELS-associated changes in posterior cingulate FC have also been reported in human subjects examined during the resting state (Graham et al., 2014; Philip et al., 2013a).

ELS animals compared to controls showed a greater number of significant functional connections (i.e. degree centrality) in the pain circuit and associated regions, including in particular the anterior and posterior insula, amygdala (La/BL), retrosplenial ('posterior cingulate') cortex, lateral septum and stria terminalis (Fig. 5). Of note, while ELS resulted in an increase in degree centrality in the lateral/basolateral amygdala, we noted a decrease in degree centrality in the central nucleus of the amygdala. Such divergent functional outcomes for these amygdalar nuclei have been previously reported in rodent stress paradigms by others (Connell et al., 2006; Ortiz et al., 2007; Reznikov et al., 2007; Shors and Mathew, 1998).

Also of interest was our finding that function of the sensory thalamus was altered by ELS, with decreases noted in rCBF, as well as a marked decrease in degree centrality. The ventroposterior medial and ventroposterior lateral nuclei (VPM, VPL) have been

implicated in some forms of sensory gating within corticothalamic and thalamocortical pathways (Castro-Alamancos, 2004; Kim et al., 2003), with the VPL having been reported to show graded electrophysiologic responses to graded CRD pressures (Yang et al., 1998, 1999).

In summary, ELS compared to controls resulted in increased rCBF in medial prefrontal cortical regions (Cg1, PrL) and decreased rCBF in the amygdala. At the same time, ELS animals showed a shift of the FC (negative correlation) of the amygdala with the anterior cingulate, characteristic of controls, to a more posterior region of the cingulate. Within regions of the pain circuit, ELS resulted in increased FC of the lateral/basolateral amygdala, but a loss of FC for the sensory thalamus. Together, these results suggest that ELS alters functional activation of the thalamo-cortico-amydala pathway.

4.4. Sex differences in functional brain responses to CRD

In our study, a significant 'Sex' effect on rCBF was noted across a number of regions implicated in pain processing, including the anterior cingulate, retrosplenial, prelimbic, and primary somatosensory cortices, as well as the amygdala (Ce, La/BL, Me), periaqueductal gray, and sensory thalamus. A significant 'Sex' effect was noted also in related limbic/paralimbic regions, including the hippocampus, median raphe, and caudate-putamen (Fig. 6, column 3). Regions noted above as showing sex differences were largely identical to those showing sex differences in our earlier study in ELS-naive animals that compared functional brain activation to acute CRD (60-mm Hg) with no-CRD (0-mm Hg) (Wang et al., 2009). In the current study, females compared to males (both ELS and controls) showed greater rCBF in retrosplenial cortex, ectorhinal/temporal association cortex, the amygdala, dentate gyrus, median raphe, and thalamus (Hb, Rt), while a modest significant decrease in rCBF was noted in the anterior-ventral cingulate, anterior insula, periaqueductal gray, thalamus (MD, Po) and mediodorsal caudate-putamen. The fact that we detected significant sex differences on functional brain mapping but not on the VMR response, suggests that functional neuroimaging may assess the multidimensional nature of pain, including its sensory and affective components, more broadly than behavioral measures alone.

4.5. 'Sex x ELS' interaction in functional brain responses to CRD

A significant 'Sex x ELS' interaction was apparent in the pain and limbic/paralimbic regions, including the anterior-dorsal cingulate, prelimbic, orbital cortex, and portions of primary somatosensory cortex (S1FL), as well as in the amygdala (La, Ce), locus coeruleus/lateral parabrachial nucleus, anterior-dorsal pretectal nucleus, and thalamus (VPL/VPM, CM, Hb) (Fig. 3, column 4).

ELS exposure in females (ELS/female vs. CON/female) compared to that in males (ELS/male vs. CON/male) showed a greater increase in rCBF particularly in the locus coeruleus/lateral parabrachial nucleus (Fig. 2, AP -9.7 mm). Seed analysis revealed a significantly increased positive correlation of the locus coeruleus/lateral parabrachial nucleus with the amygdala in ELS-females, whereas the other groups showed a negative or absent functional connectivity (Fig. 7, AP -9.7 mm). Our results are consistent with prior evidence for a sexual dimorphism of the locus coeruleus (Luque et al., 1992; Pinos et al., 2001), as well as sex differences in the stress responsiveness of the brain noradrenergic system (Curtis et al., 2006). Sex differences in brain functional responses to CRD have been previously reported in the parabrachial nucleus (Murphy et al., 2009; Wang et al., 2009), amygdala, cingulate (Lu et al., 2004; Naliboff et al., 2003), and locus coeruleus complex (Wang et al., 2009). Our results extend this work by suggesting that ELS differentially acts at the locus coeruleus/lateral parabrachial nucleus in a sexually dimorphic manner. Of related relevance to our findings is prior work by Labus et al. that demonstrated sex differences in IBS subjects in the effective connectivity of the emotional-arousal network (locus coeruleus complex, amygdala, rostral and subgenual cingulate areas) during expected and delivered CRD (Labus et al., 2008). Though ELS was not specifically examined, women IBS subjects showed consistently strong positive connectivity between amygdala and locus coeruleus complex, whereas men showed weaker, and sometimes negative connections.

In addition, a significant 'Sex x ELS' interaction was noted in related limbic/paralimbic regions, including the lateral septum, hypothalamus, bed nucleus of the stria terminalis, hippocampus, thalamus (Hb, Rt), as well as the caudate-putamen, external globus pallidus, and the cerebellum. ELS-related alterations of function of these regions have been reported previously by others (e.g. (Brydges et al., 2013; Coplan et al., 2010; Hui et al., 2011; Lippmann et al., 2007; Lukas et al., 2011; McCrory et al., 2012; Suzuki et al., 2014; Vicentic et al., 2006)). The 'Sex x ELS' interaction was particularly prominent in the globus pallidus (Fig. 6, column 4, AP -1.2 mm). The globus pallidus is well known to play a role in nociception and pain (Chudler and Dong, 1995), with alterations reported in functional connectivity of this structure within the pain network in IBS patients (Ellingson et al., 2013), as well as in those with fibromyalgia (Cifre et al., 2012). Recent work has demonstrated an association of ELS with altered functional responses of the globus pallidus during an emotional challenge (Suzuki et al., 2014). Our results extend prior work by suggesting that ELS differentially acts at the globus pallidus, hypothalamus, raphe, septum, caudate-putamen and cerebellum in a sexually dimorphic manner.

4.6. Limitations

There are several potential limitations to our study. (1) Although we were able to show clear ELS- and sex-related differences in the functional brain response, there are limitations to exploring these at a single level of CRD (60-mm Hg), and future work will need to examine responses at intermediate levels of distension; (2) There were no significant group differences in postmortem uterine weights (personal communication). A more in-depth approach to assessing possible effects of female sex hormones on brain responses might have incorporated serum estradiol levels; (3) Given that the timing of ELS may be an important variable in determining the adult phenotype, care needs to be taken in extrapolating our results in the neonatal limited bedding rodent model to that of IBS human subjects with a history of ELS, in particular since certain stressors applied at specified times may actually result in resilience against stress and other adversities later in life (Bock et al., 2014).

4.7. Conclusion

In the rodent model, altered neonatal maternal care elicited visceral hyperalgesia in later life. ELS resulted in significant changes in functional activation during CRD of numerous regions within the pain pathway. In particular, ELS compared to controls resulted in increased rCBF in medial prefrontal cortical regions and decreased rCBF in the amygdala. ELS resulted in increased FC of the lateral/basolateral amygdala, but a loss of FC for the sensory thalamus. Sex differences in rCBF were present, though less broadly expressed. Significant sex differences were noted at the level of the cortex (prefrontal, primary somatosensory, retrosplenial), amygdala, dorsal hippocampus, raphe, sensory thalamus, and caudate-putamen. A significant 'Sex x ELS' interaction was apparent most notably in the locus coeruleus/lateral parabrachial nucleus and globus pallidus. Our results suggest that ELS alters functional activation of the

thalamo-cortico-amydala pathway, as well as the emotional-arousal network (amygdala, locus coeruleus), with evidence that ELS may additionally show sexually dimorphic effects on brain function.

Conflict of interest

No competing financial interests or potential conflicts of interest.

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